

Antifungal efficacy of clove flower buds (*Syzygium aromaticum*) oil and aqueous extract against dermatophytes clinical isolates

To Cite:

Shalayel MHF, Nour S, Al-Mazaideh GM, Mahmoud SY, Farrag ES. Antifungal efficacy of clove flower buds (*Syzygium aromaticum*) oil and aqueous extract against dermatophytes clinical isolates. *Medical Science*, 2021, 25(109), 744-750

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Peer-Review History

Received: 25 February 2021
 Reviewed & Revised: 26/February/2021 to 19/March/2021
 Accepted: 20 March 2021
 Published: March 2021

Peer-review Method

External peer-review was done through double-blind method.

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ABSTRACT

This study aimed to define the efficacy of clove's oil and its aqueous extract against dermatophytes clinical isolates in comparison to some antifungal drugs namely, fluconazole, ketoconazole, itraconazole, terbinafine and griseofulvin, by using the in vitro susceptibility test. Terbinafine was found to have the highest potency against all isolates, with an MIC median range of 0.002-0.5 µg/mL. Itraconazole, ketoconazole and griseofulvin revealed comparable good antifungal potency in terms of MIC, 0.05 - 0.5 µg/mL, 0.25 - 0.5 µg/mL, and 0.5 - 1 µg/mL respectively. fluconazole displayed the least antifungal efficacy (2 - 8 µg/mL). MIC results for *Syzygium aromaticum* oil and clove aqueous extract were 1.25-2 µg/mL and 125 µg/mL respectively. It was concluded that *Syzygium aromaticum* oil exhibited a significant antifungal efficacy while, the aqueous extract did not reveal such antifungal potential. This may be due to the hydrophobicity of most of the active ingredients.

Keywords: Clove; *Syzygium aromaticum*; Dermatophytes; Susceptibility testing; Terbinafine.

1. INTRODUCTION

Fungal infection is one of the most widespread infections throughout the world. In human being it happens when the invading fungus prevails body area and is much enough to exceed handling capability by the immune system. A new spectrum of human fungal infections is rising due to increased cancer, AIDS, and immunocompromised patients. Cutaneous fungal infections are superficial infections typically affecting the skin, hair, and nails (Charles, 2009). Most predominantly, these fungal infections are typically caused by dermatophytes, however they might even be caused by non-dermatophyte fungi and yeast like candida species (Goldstein et al., 2000; Clinard & Smith, 2015; Farhan et al., 2017). The term dermatophyte refers to a fungal organism



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that causes tinea (Ely et al., 2014). Hence, dermatophytoses are known as tinea or ringworm infections, which are further classified by the region of the body infected (Farhan et al., 2017). Since they need keratin for growth, dermatophytoses are particularly confined to the stratum corneum, hair shafts and nails. Infections are typically caused by three genera of dermatophytes namely: Trichophyton, Epidermophyton, and Microsporum. In the U.S., Trichophyton is the most prevalent genus accounting for more or less 80% of dermatophytosis (Vander Straten et al., 2003).

The resistance of some fungi species to the current antifungal therapeutics and the adverse effects as well as costiveness, had motivated researchers to look for alternative natural compounds (Rana et al., 2011). In contrast to bacteria, fungi are eukaryotes, and consequently most agents toxic to fungi are also toxic to the patient. Experimental attempts for discovery of new drugs and dietary supplements derived from plants have been recently accelerated. Microbiologists, ethno-pharmacologists, botanists, and natural-products chemists are sieving the earth for phytochemicals and “leads” which could be progressed for treating infectious diseases (Mohammadi et al., 2014). Herbal medicinal products have been authenticated as a leading source for disclosing new pharmaceutical compounds that have been utilized to address serious diseases. Many herbs have been declared to have pharmacological activities attributable to their phytoconstituents such are glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes, etc. Various plant extracts have great potential against infectious agents and can be used for therapeutic purposes (Shalayel et al., 2017).

Syzygium aromaticum (clove) possesses diverse pharmacological potentials and is widely utilized as a traditional spice and food preservative (Batiha et al., 2020). Being as a carminative, cloves are traditionally used to increase gastric hydrochloric acid and to ameliorate peristaltic motion of the gut. In addition, the cloves are antimutagenic, anti-inflammatory, antioxidant, antiulcerogenic, antithrombotic and antiparasitic, antibacterial and anti-inflammatory (Pandey & Singh, 2011). Many antimicrobial activities were seen for *Syzygium aromaticum* seed. It induced oxidative stress in *E. coli*, *P. aeruginosa* and *S. aureus*.

This study is aimed to determine the efficacy of clove oil and its aqueous extract against dermatophytes in comparison to some antifungal drugs which used commonly in treatment of fungal infection by using the *in vitro* susceptibility test.

2. MATERIALS AND METHODS

Microbial assay was carried out in a fairly established microbiology laboratory (College of Medicine, Nile University, Sudan). All over the study was carried out in the period from January 2018 to January 2019. This study is an experimental study on consecutive samples received in the mycology laboratory from 100 patients clinically suspected of dermatophytosis.

Processing and handling of clinical isolates

The clinical samples including skin, hair and nail were collected from 100 patients for fungal identification and antifungal susceptibility testing. The study was ethically approved by University of Bahri Research Ethical committee. All patients were assured that all their obtained information will be handled in a confidential atmosphere and it will not affect their life after taking written consent. Neither additional clinical history nor patients' follow-up was considered.

In vitro susceptibility testing

Agar-based disk diffusion assay for antifungal susceptibility testing of dermatophytes was performed according to the Clinical Laboratory Standard Institute (CLSI) guidelines (Wayne, 2008).

Antifungal agents

The study included 5 antifungal drugs and clove oil. Antifungal drugs powders were purchased from Hunan Insens Biotech Co., LTD (China). The antifungal drugs included: fluconazole, ketoconazole, itraconazole, terbinafine and griseofulvin.

Aqueous solutions were prepared with distilled water for fluconazole and with 100% dimethyl sulfoxide for the other tested antifungal drugs. Concentrations of the final stock dilutions were ranged from 0.03 to 16 µg/mL for ketoconazole, terbinafine and itraconazole while they were 0.03 to 8 µg/mL for griseofulvin and 0.125 to 64 µg/mL for fluconazole.

For clove oil, pure and sterile clove oil was purchased from Amriya Pharm Industries Pharmaceutical Company in Alexandria, Egypt, with a precaution; bottle not to be opened before test. It was reduced to 10% of its original volume to be used in antifungal susceptibility testing. For preparation of clove buds aqueous extract, 1 gram of minced clove buds was soaked in 1 L of distilled water and left for 24 hours then filtered to be ready for susceptibility testing. The plant was identified by a Pharmacognosy specialist.

Test procedure

Dermatophytes inoculum suspensions were typically prepared after culturing for seven days on Sabouraud agar at room temperature (~28°C). Dermatophytic colonies had been submerged roughly with 10 mL of distilled water; and the end of a sterile loop was used to scrap the surface to attain the suspensions. The ensuing mixture of hyphal fragments and conidia had been transferred to sterile tubes after being withdrawn and left at room temperature for approximately 30 minutes to get the heavy particles being sedimented. Fungal isolates were subjected to antifungal susceptibility testing using the agar-based disk diffusion test (Kirby-Bauer Method). Disks containing the test agents were applied to the surfaces of inoculated plates. MIC was indicated by the highest dilution that suppressed fungal growth (Ghannoum et al., 2004).

Endpoint determination

Values of endpoint determination were visionally proceeded every 24 h until we got a typical fungal growth in the control drug-free wells. For azole agents, griseofulvin and clove oil, the MIC was determined as the lowest concentration of antifungal drugs that caused 80% - 100% inhibition of growth (Santos et al., 2006).

Statistical Analysis

This is a descriptive study and the MIC was determined by defining the lowest concentration of clove oil and clove aqueous extract that clearly inhibited visible growth of dermatophytes.

3. RESULTS

A total of 100 samples were collected from the patients including skin, hair and nail. Out of which, 39 (39%) samples were found to be positive for dermatophytes and 61 (61%) were negative. By using standard colony morphological and microscopic characteristics, under supervision of a senior microbiologist, fungal isolates from the 39 positive culture samples included both dermatophytes, with predominance of *T. verrucosum* followed by *T. soudanense*, *T. mentagrophytes*, *M. Canis* and non-dermatophytes especially *Aspergillus sp.* which could be easily identified by morphological characteristics.

Colonies of *T. verrucosum* are white to cream-coloured; slow growing, small, button-shaped, with a suede-like to velvety surface, an elevated centre, and flat periphery with some submerged growth. The ends of some hyphae are club-shaped, and sometimes divided, presenting the so-called "antler" effect. Colonies of *T. soudanense* are slow-growing with a flat to folded, suede-like surface. Overwhelmingly, there is a submerged growth at fringes. Surface mycelium is characteristically a deep orange in colour. Microscopically, the hyphae often display right-angle or reflexive branching. Colonies *T. mentagrophytes* are generally flat, white to cream in colour, with a powdery to granular surface. Some cultures showed central folding or developed raised central tufts or pleomorphic suede-like to downy areas. Microconidia were hyaline, smooth-walled, and were predominantly spherical to subspherical in shape. The SDA culture was able to detect 39 cases (39%) from the 100 clinically suspected cases of skin, hair and nail dermatophytosis. Out of 100 clinical isolates, 83 samples (83%) were positive in KOH mount and hence microscopic examination. Thirty samples (30 %) were positive in both the microscopic examination and culture, 9 samples (9%) were negative in microscopy but culture positive (Table 1).

Overall, irrespective of the dermatophytes 'isolates, terbinafine was the most potent antifungal against all isolates, with an MIC median range of 0.002-0.5 µg/mL, followed by itraconazole (0.05 - 0.5 µg/mL), ketoconazole (0.25 - 0.5 µg/mL), and griseofulvin (0.5 - 1 µg/mL), while fluconazole recorded the least potent antifungal efficacy (2 - 8 µg/mL). In this context, *Syzygium aromaticum* oil showed a reasonable antifungal efficacy in terms of MIC (1.25-2 µg/mL) but, aqueous extract did not display such efficacy as revealed in Table 2.

Table 1 Findings of potassium hydroxide mount and culture among positive samples

KOH and Culture Observation among positive samples		
Findings	Number of patients	percentage
KOH Positive, Culture Positive	30	30 %
KOH Positive, Culture -Negative	53	53 %
KOH Negative, Culture Positive	9	9%
KOH Negative, Culture -Negative	8	8%
Total cases	100	100%

Table 2 Susceptibilities of dermatophytes to the tested antifungal drugs, clove oil and clove aqueous extract

	Fluconazole 0.125 to 64 µg/mL MIC Median	Ketoconazole 0.03 to 16 µg/mL MIC Median	Itraconazole 0.03 to 16 µg/mL MIC Median	Terbinafine 0.03 to 16 µg/mL MIC Median	Griseofulvin 0.03 to 8 µg/mL MIC Median	Clove oil	Clove aqueous extract
<i>Trichophyton verrucosum</i>	4	0.5	0.05	0.002	0.5	1.25	125
<i>Trichophyton soudanense</i>	4	0.5	0.05	0.002	0.5	1.25	125
<i>Trichophyton mentagrophytes</i>	2	0.25	0.05	0.004	0-5	1.25	125
<i>Microsporum canis</i>	8	0.25	0.05	0.002	0.75	1	125

4. DISCUSSION

Dermatophytosis is a commonly encountered superficial fungal infection in the tropical and subtropical countries (Basak et al., 2019). Dermatophytic infections are being acquainted at a frightening rate in Africa, particularly within the northern geographical zone. This is mostly due to environmental and socioeconomic conditions, some local cultural habits, lack of trustworthy diagnostic facilities and staff as well as inefficient treatment (Nweze & Eke, 2016). In the near past, a global rise in the occurrence of fungal diseases has been monitored. Furthermore, the resistance to some antifungal drugs used in clinical practice had dramatically increased. The majority of the utilized antifungal medications have diverse adverse effects in terms of efficacy, toxicity and cost as well as their frequent use. All these players led to emerging resistant strains. For this reason, there may be a cogent need for evolving an antifungal that selectively acting on unprecedented targets and pertinent to a wide range of structural categories.

Herbal products, either as natural refined phytochemicals or as crude plant extracts, provide boundless opportunities for novel drug discovery owing to their matchless structural diversity (Mishra & Kaur, 2020). Plants and their bioactive ingredients, sometimes called secondary metabolites, confer reproducible opportunities for improving animals' production by their inclusion in the food. Several publications have reported the presence of antifungal potential for some compounds in plant. Nevertheless, systematic reviews and literature on the natural phytoconstituents as alternatives to the routinely used antifungal medications are still insufficient. Taxonomic distribution and chemical classes are the basis for determining the distribution of these antifungal compounds. The antifungal natural compounds are belonging to some substantial categories of secondary metabolites liketerpenoids, phenolics, sterol, flavonoids, saponins, alkaloids, peptides, and proteins (Mishra & Kaur, 2020).

Regarding susceptibility test, our results, irrespective of the dermatophytes' isolates, revealed that terbinafine was the most potent antifungal against all isolates, followed by itraconazole, ketoconazole, and griseofulvin while. Fluconazole recorded the least potent antifungal efficacy. Macro broth dilution and micro broth dilution methods are generally laborious and need expertise to be carried out in laboratories compared to the antimicrobial susceptibility testing by disc diffusion and 'E' test method (Dogra et al., 2019). In accordance to the standard guideline of CLSI, disc diffusion test and E-test are not typically recommended for dermatophytes antifungal susceptibility testing (Aktas et al., 2014). Nevertheless, disc diffusion test is the most utilized method in this issue and studies remain comparing disc diffusion with broth microdilution methods. Jessup et al. (2000) determined the antifungal susceptibilities of dermatophytes to fluconazole, griseofulvin, itraconazole, and terbinafine. With regard to the other agents tested, terbinafine had the highest antifungal activity against all of the dermatophytes followed by itraconazole and then griseofulvin.

Some comparable results were reported by Shalaby and co-researchers. The most effective antifungal potentiality was for Clotrimazole, followed by Miconazole (Shalaby et al., 2016). Variations in susceptibility testing and predominant dermatophytes' species may give an idea about the overlap between the anthropophilic species and the zoophilic species (Aboueisha & El-Mahallawy, 2020).

In terms of MIC, *Syzygium aromaticum* oil showed a reasonable antifungal efficacy. However, clove buds aqueous extract did not reveal such antifungal potential. This may be explained by the hydrophobicity of most of active ingredients (Burt, 2000; Pasqua et al., 2007). Our finding is consistent with a study carried out by Eman and El-Diasty who reported that Clove oil had in vitro strong antifungal efficacy against Dermatophytes spp (Rana et al., 2011). Some active ingredients in many medicinal herbs including clove (*Syzygium aromaticum*), like eugenol, exert their antifungal effects on the cell wall and cell membrane of *T. rubrum*. Eugenol acts on cell membrane by a mechanism that likely embrace the inhibition of ergosterol biosynthesis. The lower ergosterol content interferes

with the integrity and functionality of the cell membrane (Pereira et al., 2013). Eugenol showed strong antifungal activity against *T. rubrum* as the MIC values of which were lower than 500 µg/ml (Martinez-Rossi et al., 2017).

Yassin et al., (2020) reported that the ethyl acetate extract of *S. aromaticum* exhibited a potent antifungal activity against *C. albicans*, *C. glabrata*, and *C. tropicalis* at low concentrations and can be utilized as a natural antifungal drug. Moreover, Shalayel et al., (2021) showed that Stigmasterol and Campesterol, two active ingredients of clove oil, can effectively inhibit both candidal α -glucanases (3N9K and 3O6A) and their association with commercial antifungal therapeutics used in candidiasis treatment (Shalayel et al., 2021). Aiensaard & Punareewattana, (2017) used three essential oils extracted from *Syzygium* for investigation of their antifungal potentials against *Trichophyton mentagrophytes*, *Microsporum canis*, and *Microsporum gypseum*.

The results of their analysis displayed that eugenol and its derivatives were the major ingredients possessing antifungal efficacy (Shalayel et al., 2017). In addition to their antimicrobial potentiality, essential oils have several advantages. The first one, they could help mitigate inflammation as they exhibit antioxidant activity. Secondly, essential oils are derived from medicinal plants and contain mixture of active ingredients which may result in additive or synergistic influences or having potential at multiple targets. Thirdly, modes of antifungal resistance are commonly related to receptor-mediated interaction of the antifungal drugs. Thus, hydrophobicity of essential oils that cause interference with cell membrane domain structure may subdue the mechanism of fungal resistance. Lastly, essential oils are advantageously having minimal toxicity when utilized for the skin. However, irritation is the most predominant adverse effect for topical application (Amorati et al., 2013; Bassolé & Juliani, 2012; Solórzano-Santos & Miranda-Novales, 2012). Consequently, essential oils may become the provenance of new therapeutic molecules and their incorporation into topical formulas is an effective, safe, and alternative for the treatment for dermatophytes including onychomycosis. Since essential oils are affluent in substances with low molecular weight, their active ingredient could attain improved penetration enabling fungal elements to access some areas that could not be reached by other topically used drugs (Flores et al., 2016).

5. CONCLUSION

Terbinafine was the most potent antifungal against all isolates, followed by itraconazole, ketoconazole, and then griseofulvin. Whereas, fluconazole recorded the least antifungal potency. *Syzygium aromaticum* oil displayed a significant antifungal efficacy while, the aqueous extract did not reveal such antifungal potential. This may be due to the hydrophobicity of most of the active ingredients. A limitation of our study is that we evaluated the anti-fungal activity of the crude clove oil and aqueous extract but not the alcoholic and various organic solvents' extracts of clove buds as well as the known active ingredients. However, our results may serve as a guide for future in vivo studies of the clinical utilization of clove oil and clove ingredients in treating candidal and dermatophyte infections.

Authors' contributions

This work was carried out in collaboration among all authors. Author Mohammed H. F. Shalayel and Saada Nour designed the study, performed the experimental part, wrote the protocol, and wrote the first draft of the manuscript. Author Ghassab M. Al-Mazaideh, Sabry Younis Mahmoud and Eman Saleh Farrag managed the literature searches of the study. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Ethical approval and patient consent

The study was ethically approved by University of Bahri Research Ethical committee (UBREC-CM-17-05).

Funding

This paper received no external funding.

Abbreviations

Acquired Immune Deficiency Syndrome	AIDS
Clinical Laboratory Standard Institute	CLSI
Minimum Inhibitory Concentration	MIC

Data and materials availability

All data associated with this study are present in the paper.

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